Basic presynaptic functions in hippocampal neurons are not affected by acute or chronic lithium treatment

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Presynaptic functions in hippocampal neurons are not affected by acute or chronic lithium treatment.

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Abstract

Lithium is an effective mood-stabilizer in the treatment of bipolar affective disorders. While glycogen synthase kinase 3-mediated and inositol depletion-dependent effects of lithium have been described extensively in literature, there is very little knowledge about the consequences of lithium treatment on vesicle recycling and neurotransmitter availability. In the present study we have examined acute and chronic effects of lithium on synaptic vesicle recycling using primary hippocampal neurons. We found that exocytosis of readily releasable pool vesicles as well as recycling pool vesicles was unaffected by acute and chronic treatment within the therapeutic range or at higher lithium concentrations. Consistent with this observation, we also noticed that the network activity and number of active synapses within the network were also not significantly altered after lithium treatment. Taken together, as lithium treatment does not affect synaptic vesicle release at even high concentrations, our data suggest that therapeutic effects of lithium in bipolar affective disorder are not directly related to presynaptic function.

Introduction

Bipolar disorder is a severe mental disease with a typical onset during early adulthood and a lifetime prevalence estimated to 4% within the United States (Kessler, Berglund et al. 2005). The symptoms are characterized by recurrent episodes of elevated mood and depression, termed mood episodes (Anderson, Haddad et al. 2012). During these episodes, extreme changes in activity and energy go along with behavioral abnormalities. Interestingly, even 60 years after initial studies by John Cade (Cade 1949), lithium remains a first line monotherapeutic agent in today's treatment strategies (Goodwin 2009).

Despite the many years of clinical application, the molecular mechanism underlying lithium effects

remains largely unknown. Several studies have shed light on potential mechanisms involving the glycogen synthase kinase 3 (GSK3)-signaling, both at the presynaptic and postsynaptic sites (Goold, Owen et al. 1999; Hall, Lucas et al. 2000; Tyagarajan, Ghosh et al. 2011). The generation and characterization of Myshkin mice has identified Na^+/K^+-ATPase as another target for lithium. Interestingly, blocking the extracellular-regulated kinase (ERK) or treating with lithium rescued the manic behavior in these mice (Kirshenbaum, Clapcote et al. 2011), implicating ERK signaling in lithium-mediated effects. A recent study has described signaling cross talk between ERK and GSK3β pathways in regulating GABAergic transmission via gephyrin phosphorylation at S268 and S270 respectively (Tyagarajan, Ghosh et al. 2013). Furthermore, lithium also decreases glutamate receptor expression levels (Du, Gray et al. 2004). Hence, it is likely that the postsynaptic effect of lithium is mediated via Na^+/K^+-ATPase and activation of ERK, GSK3β pathways to facilitate hetero-synaptic adaptations and mediate network activity changes.

It is still unclear whether lithium-mediated adaptations can be exclusively attributed to those postsynaptic mechanisms described or whether also presynaptic changes contribute to lithium-mediated effects. A lithium induced inhibition of kinesin light chain phosphorylation by GSK-3(Morfini, Szebenyi et al. 2002) impacts axonal transport and could ultimately result in modification of synaptic vesicle populations upon chronic treatment. Another possible presynaptic mechanism could be via the reduction of brain inositol levels (Allison and Stewart 1971) and interfering with the PIP_2/IP_3-signaling (Berridge, Downes et al. 1989; King, Teo et al. 2009; Kimata, Tanizawa et al. 2012), which in turn affects both exo and endocytosis (Milosevic, Sorensen et al. 2005; Antonescu, Aguet et al. 2011; van den Bogaart, Meyenberg et al. 2011; van den Bogaart, Meyenberg et al. 2012).

Presynaptic vesicular exocytosis has also been described to be accelerated due to enhanced Ca+-influx when high doses of lithium are applied in expense of sodium (Reuter and Porzig 1995). Interestingly, asynchronous neurotransmitter release is facilitated under such condition (Otsu, Shahrezaei et al. 2004).

Given the lithium-induced activation of ERK and its role in GABA release (Cui, Costa et al. 2008), and the activation of PIP_2/IP_3 signaling for vesicular recycling, we hypothesized that one of the lithium-induced effects could be via changes in synaptic vesicle recycling. In support of this idea it has been demonstrated earlier that high concentrations of fluoxetine (5 mM), another antidepressant, inhibits glutamate exocytosis from nerve terminals (Wang, Su et al. 2003). In addition, functional cellular imaging shows that antipsychotics such as chlorpromazine and clozapine, inhibit exocytosis (Tischbirek, Wenzel et al. 2012), thus highlighting the importance of potential presynaptic pathologies in psychiatric diseases.

Several groups have shown that optical methods, such as the pH-sensitive fusion-protein synaptopHluorin (Miesenbock, De Angelis et al. 1998; Sankaranarayanan, De Angelis et al. 2000), the pH-indicating dye CypHer5 coupled to an antibody against synaptotagmin-1 (Adie, Kalinka et al. 2002) and styryl dyes (Betz and Bewick 1992) are suitable for kinetic analysis of exo- and endocytosis and determination of the vesicle pools as well as network activity (Welzel, Tischbirek et al. 2010).

In the present study, we sought to determine the functional impact of lithium treatment on synaptic vesicle recycling and therefore the availability of various neurotransmitters for their action. Using in vitro live-cell microscopy, we examined both the exo- and endocytosis in synaptopHluorin transfected

neurons, and anti-synaptotagmin1-CypHer5-labeled vesicles in primary hippocampal neurons treated with lithium. While previous reports are based on varying concentrations ranging from about 1-2 mM (Naccarato, Ray et al. 1974; Hallcher and Sherman 1980) to 5-20 mM (Goold, Owen et al. 1999; Hall, Lucas et al. 2000; Williams, Cheng et al. 2002), the therapeutically relevant lithium plasma concentration is 0.5-0.8 mM (Kerwin 1999; Srisurapanont, Pratoomsri et al. 2000).

We therefore treated cells with both acute (10 or 40 mM) and chronic (1 mM) concentrations and found that under either treatment paradigm lithium did not affect exo- and endocytosis in terms of release probability or uptake kinetics. We also found that spontaneous vesicle turnover was not affected by lithium treatment. This data is supported by FM4-64 experiments, which suggest that lithium very likely does not alter the vesicle pool size during development. Further analysis confirmed rather stable recycling pool sizes. Based on the evidence presented it is unlikely that lithium action at the presynaptic axon terminals are via alterations in the kinetic properties of the recycling vesicles.

Materials and methods

Ethics statement

All animals were handled in accordance with good animal practice as defined by the guidelines of the Free State of Bavaria, and all animal work was approved by the Kollegiales Leitungsgremium of the Franz-Penzoldt Zentrum, Erlangen (reference number TS-1/ 10).

Cell culture and transfection

Hippocampal neuronal cultures were prepared as described in (Tischbirek, Wenzel et al. 2012). Neurons were transfected on DIV3 (days in vitro) with synaptopHluorin (Sankaranarayanan, De Angelis et al. 2000) under control of a synapsin promoter with a modified calcium phosphate method as described (Threadgill, Bobb et al. 1997). Experiments were performed between DIV15 and DIV40. Cell viability was assured during the course of experiments by lactate dehydrogenase activity measurements (CytoTox96® Non-Radioactive Cytotoxicity Assay, Promega, Mannheim).

Imaging and Data Analysis

Experiments were conducted at room temperature on a Nikon TI-Eclipse inverted microscope equipped with a 60x, 1.2 NA water immersion objective and Perfect Focus System™. Fluorescent dyes were excited by an Intensilight C-HGFI (Nikon) through excitation filters centered at 482 nm, 561 nm, and 628 nm using dichroic long-pass mirrors (cutoff wavelength 500 nm, 570 nm, and 660 nm), respectively. The emitted light passed through emission band-pass filters ranging from 500 nm to 550 nm, 570 nm to 640 nm, and 660 nm to 730 nm, respectively (Semrock, Rochester, NY) and was projected onto a cooled electron-microscopy (EM) charge-coupled device camera (iXonEM DU-885 and iXonEM DU-897; Andor Technology, Belfast, Ireland).

Cover slips were placed into a perfusion chamber (Volume = 500 µl) containing saline (144 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl2, 2.5 mM MgCl2, 10 mM Glucose, 10 mM Hepes, pH 7.5). In the lithium-experiments, sodium-chloride was substituted equimolarily with lithium-chloride to achieve final concentrations of 1, 10 or 40 mM lithium in the solution. The steady-state level of lithium concentration

was monitored during long-term experiments by analyzing cell supernatants on a Roche COBAS INTEGRA™ ISE direct module.

In the synaptopHluorin-experiments synaptic boutons were stimulated by electric field stimulation (platinum electrodes, 10 mm spacing, 1 ms pulses of 50 mA and alternating polarity); 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Bioscience) and 50 µM DL-2-Amino-5-phosphonopentanoic acid (DL-AP5, Tocris Bioscience) were added to prevent recurrent activity. The solution was exchanged in the acute treatment paradigm experiment by a piezo-controlled stepper device (SF-77B, Warner Instruments). Throughout all experiments perfusion was kept constant at 0.2 ml/min (Fast-Step Valve Control Perfusion System VC-77SP8, Warner Instruments).

Exocytosis of synaptic vesicles from the readily releasable pool (RRP) was induced by electrical stimulation with 50 pulses at 10 Hz in synaptopHluorin-transfected neurons (Murthy and Stevens 1999).

The total recycling pool was labeled with FM-4-64 (Invitrogen, Karlsruhe), which was taken up by compensatory endocytosis after a stimulation with 1200 pulses at 30 Hz (Ryan and Smith 1995). Complete destaining, as a measure of total amount of vesicles, was achieved by a threefold stimulation of 900 pulses at 30 Hz (Groemer and Klingauf 2007).

Image stacks were recorded and used to automatically detect spots of synaptic bouton size (Sbalzarini and Koumoutsakos 2005) where AP-evoked fluorescence increase (for synaptopHluorin) occurred in difference images. SynaptopHluorin fluorescence intensity was normalized to the mean stimulation dependent difference in fluorescence (ΔF) before drug application.

In the synapse labeling experiment with anti-Syt1-cypHer5 (Synaptic Systems, Goettingen), the antibody was incubated for 1 h prior to multipoint acquisition of 10x10 images per coverslip. For multipoint experiments, anti-Syt1-CypHer5 antibody-labeled synapses were detected by a Laplace-operator-based peak detection method (Dorostkar, Dreosti et al. 2010). All image and data analysis was performed using custom-written routines in MATLAB (The MathWorks Inc., Natick).

Statistical analysis

Statistical analysis was performed by MATLAB (The MathWorks Inc., Natick). Error bars indicate SEM. Treatment effects analysis was carried out by analysis of variance (ANOVA). For single group comparisons, unpaired t-tests were performed using built-in routines in MATLAB.

Results

Cell viability

To assure cell viability during the course of experiments, osmolarity was monitored and LDH-measurements were performed. Osmolarity of the medium was determined immediately before starting experiments. The obtained values remained fairly stable (mean value under lithium treatment 340.0 ± 1.0 mOsm/L versus control mean 343.7 ± 0.6 mOsm/L). Similarly, LDH-values that were assessed before starting the incubation and before the measurement on day 7 post-incubation were unaltered for control (before incubation mean activity 0.095 ± 0.012 [a.u.] and after 7 days 0.093 ± 0.011 [a.u.]) and lithium treated cells (before incubation mean activity 0.098 ± 0.023 [a.u.] and after 7 days 0.116 ± 0.010 [a.u.], student's t-test $p=0.18$, $n=9$, Figure 2D). Even the long-term treatment

yielded comparable values (mean LDH activity for lithium 0.090 ± 0.015 [a.u.] versus control 0.094 ± 0.021 [a.u.], Figure 3B), thus indicating good viability of the cells.

Acute lithium treatment

Effects of acute lithium treatment on presynaptic processes, namely exo- and endocytosis, can be monitored using pH-sensitive GFP mutants (Miesenbock, De Angelis et al. 1998) that are fused to synaptic vesicle proteins, for example the intravesicular domain of synaptobrevin-2 linked to pHluorin resulting in synaptopHluorin (spH). Emission intensities of these pH-sensing proteins increase as a consequence of deprotonation of the fluorophores by the transition from the acidic intravesicular to the more alkaline extracellular environment upon exocytosis. Fluorescence subsequently decreases during re-acidification after endocytosis.

To assess whether acute lithium treatment has an impact on synaptic vesicle release, cells were electrically stimulated with three subsequent 50 action potential-like pulse-series (AP) at 10 Hz (Figure 1A). Stimulation with 50 AP 10 Hz is a paradigm that causes release from the readily releasable pool (Murthy and Stevens 1999). Perfusion with imaging saline was followed by the same stimulation paradigm under perfusion with vehicle- (NaCl) or lithium-containing saline in concentrations of 10 and 40 mM (Figure 1A). The spH-fluorescence images, which were captured before and after stimulation (Figure 1B), were processed to generate the pseudo-colored difference-image (ΔF, Figure 1B) and fluorescence increase values relative to the baseline. Fluorescence increase values exhibited comparable values for lithium treatment with both concentrations of 10 mM (lithium mean 23.9 ± 11.8 [%] relative to control mean 24.3 ± 12.0 [%]) and 40 mM (lithium mean 25.5 ± 11.6 [%] relative to control mean 26.2 ± 11.2 [%], Figure 1C). Determined τ values, as a measure of endocytosis kinetics, were not altered upon lithium treatment (control 23.23 ± 3.33 [s] versus 10 mM lithium 21.10 ± 2.07 [s] versus 40 mM lithium 25.62 ± 8.64 [s], Figure 1A). This proves that acute lithium treatment even at high concentrations does not affect the activity-dependent exocytosis of synaptic vesicles in hippocampal neuronal cell culture.

Next, we tested whether acute lithium treatment causes a selective change in spontaneous vesicle fusion rates. We labeled spontaneously released and compensatory endocytosed vesicles in dissociated cultures of hippocampal neurons with FM 1-43 in the presence of 1 µM TTX for 30 minutes (Figure 1D, E). Subsequent application of action potential-like pulses caused exocytosis of these vesicles and a reduction of fluorescence intensities (Figure 1E). The calculated ΔF values, which provide a good estimate of spontaneous release during the incubation, show that the rate of spontaneous vesicle fusion was not significantly altered (lithium mean 809.61 ± 282.63 [a.u.] versus vehicle mean 1114.38 ± 364.61 [a.u.], Student's t-test 0.32, Figure 1F).

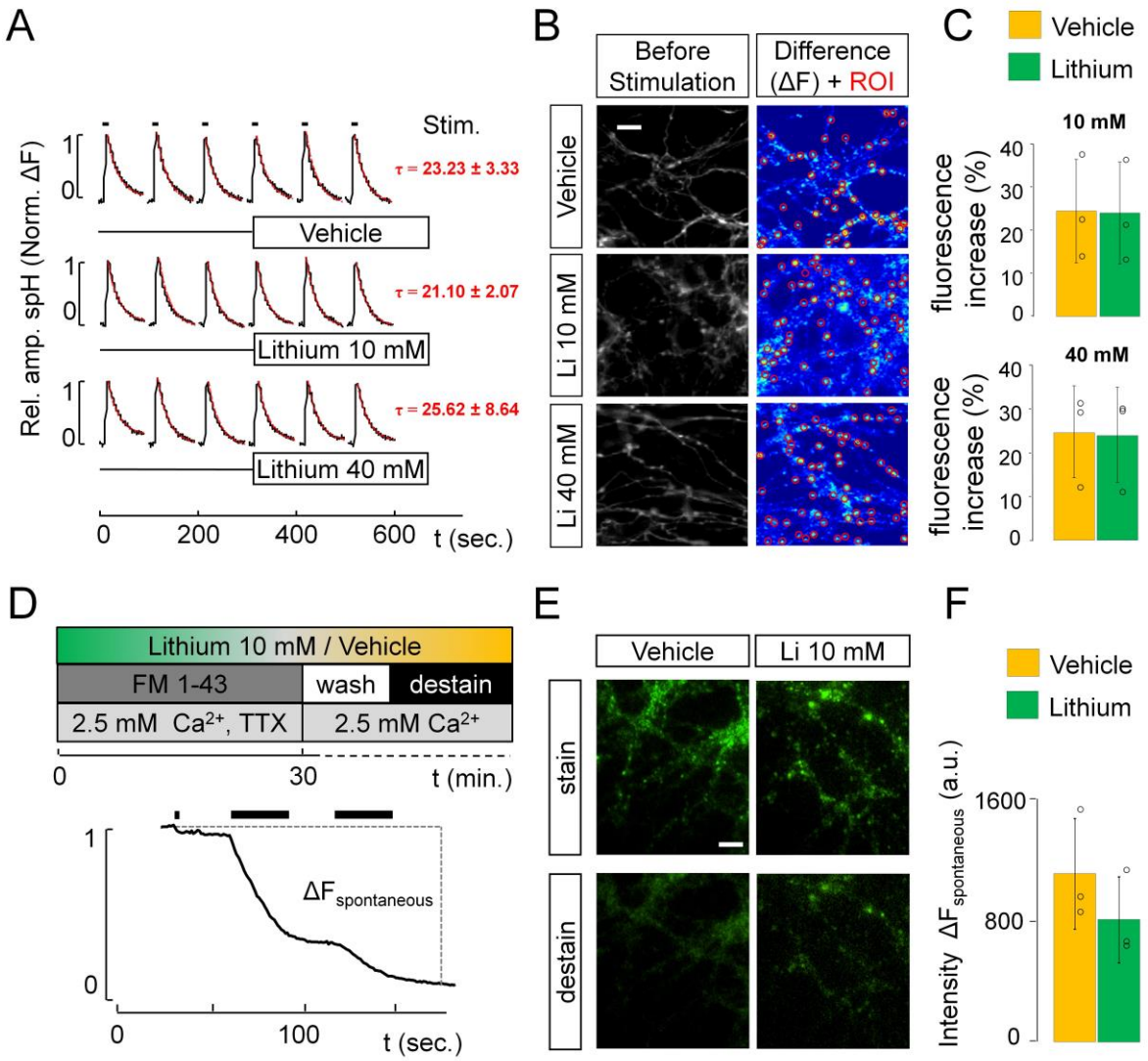

Figure 1 Acute administration of lithium has no effect on exo- and endocytosis.

A Fluorescence time courses of synapses of synaptopHluorin-transfected hippocampal neurons. Repetitive stimulation with 50 APs at 10 Hz (black bars) caused reproducible exocytosis and endocytosis kinetics. Time points and duration of perfusion shifts from imaging saline to vehicle- or lithium-containing saline are depicted by white boxes. Determined τ values (red font) indicate that the kinetics of endocytosis were unchanged upon lithium treatment. **B** Representative images of spH-transfected hippocampal neurons with processed and pseudo-colored fluorescence intensity change (ΔF) upon stimulation. The red circles mark the regions of interest that were automatically chosen and quantified. Scale bars, 10 µm. **C** Fluorescence increase upon stimulation was calculated relative to the baseline for vehicle, 10 mM and 40 mM lithium treatment. n = 3 coverslips (boutons analyzed: vehicle 706, 10 mM Lithium 641, 40 mM Lithium 635), Student's t-test: p>0.1. **D** Spontaneous fusion rates were determined after incubation of the hippocampal neurons with 2.5 µM FM 1-43, 1 µM TTX for 30 minutes at 37°C. The schematic illustration shows the experimental procedure and an example of a fluorescence-intensity-over-time-plot during the de-staining phase. **E** Representative images of FM 1-43-stained hippocampal neurons in the stained or de-stained condition. **F** Calculated ΔF values indicate that the rate of spontaneous fusions during the FM 1-43 staining phase was not altered significantly upon 10 mM acute lithium administration. n = 3 coverslips per treatment (boutons analyzed: vehicle 699, 10 mM lithium 640), Student's t-test: p>0,1. Error bars represent SD. Open circles represent individual data points.

Chronic lithium treatment

Lithium exerts its mood stabilizing effects after chronic administration and doses that lead to plasma concentrations of approximately 1 mM proved to be most efficient in terms of maximizing benefits and minimizing adverse reactions (Solomon, Ristow et al. 1996).

To test the effects of chronic lithium treatment with clinically relevant concentrations on exo- and endocytosis, spH-transfected hippocampal neurons were incubated with lithium for one week. Stimulation and analysis was carried out in a similar way as described for the acute treatment paradigm. Other than under acute application, chronic exposure to drugs can lead to structural changes of synapses (Minerbi, Kahana et al. 2009). We therefore sought to measure vesicle pool sizes by electrical field stimulation and pH-modulations as described (Li, Burrone et al. 2005; Burrone, Li et al. 2006). The fluorescence increase upon application of ammonium chloride reports the total number of spH fluorophores in synapses and has been used as an indicator of the synapse's total vesicle population (Figure 2A and B). We also calculated stimulation-evoked fluorescence increase values relative to the baseline under physiological pH and found that the fluorescence increase corresponding to the evoked vesicle release is comparable between control and lithium (lithium mean 8.35 ± 3.56 [%] versus control mean 8.29 ± 4.09 [%], Figure 2C). The NH_4^+-evoked fluorescence increase relative to the baseline, which corresponds to the total pool size, was comparable between vehicle and lithium treatment (lithium mean 97.78 ± 30.48 [%] versus control mean 118.6 ± 31.35 [%], Figure 2C). Moreover, we determined similar values for control- and lithium-treated cells in terms the total pool relative to the surface fraction as determined by the ratio of an NH_4^+ or an acid pulse-evoked fluorescence change, respectively (lithium mean 1.26 ± 0.36 versus control mean 1.51 ± 0.47). The percental release from the total pool, which was calculated as the stimulation-evoked increase in fluorescence divided by the total pool was not altered significantly (lithium mean 8.42 ± 1.56 [%] versus control mean 7.02 ± 2.82 [%]). Determined τ values were not altered upon lithium treatment (lithium mean 24.70 ± 11.09 [s] versus control mean 22.46 ± 9.49 [s]). Toxic effects that may be related to chronic treatment were ruled out by LDH activity measurements. We determined similar values for control (before incubation mean activity 0.095 ± 0.012 [a.u.] and after 7 days 0.093 ± 0.011 [a.u.], Figure 2D) and lithium treated cells (before incubation mean activity 0.098 ± 0.023 [a.u.] and after 7 days 0.116 ± 0.010 [a.u.], student's t-test $p=0.18$, $n=9$, Figure 2D). As all of the vesicle release determinants were not significantly altered, exocytosis of readily releasable pool vesicles appears unaffected by chronic lithium treatment with clinically relevant concentrations.

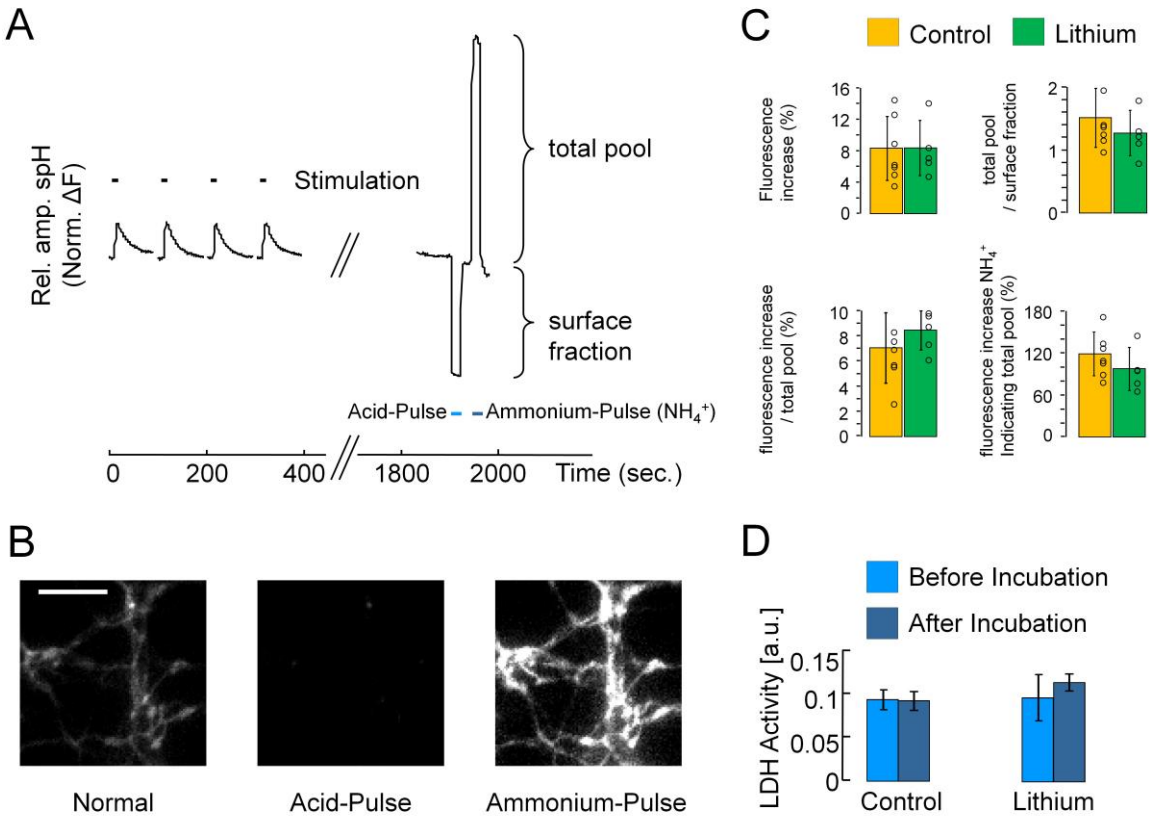

Figure 2 Chronic lithium treatment does not lead to changes in vesicle pool sizes and vesicle recycling.

A Schematic illustration of the experimental approach: cells were stimulated as indicated (black bars) with 50 APs at 10 Hz per stimulus to translocate spH molecules to the surface. After stimulation, an acid-pulse quenched all extracellular spH molecules and was followed by an ammonium-pulse, which displays the total spH pool. **B** Captured images of the stimulation paradigm shown in (A). Scale bars, 10 µm.. **C** Quantification of obtained amplitudes of spH in terms of vesicle turn-over. n = 7 coverslips for vehicle treatment (boutons analyzed: 1691). n = 5 coverslips for lithium treatment (boutons analyzed: 1119) , Errorbars represent SD. Open circles represent individual data points. Student's t-test: p>0.1. **D** LDH activity assay data that shows cell viability before and after 7 days of lithium incubation. Error bars represent SEM. n=3 experiments. Student's t-test: p>0.1.

Vesicle pool sizes and synaptic clustering under the long-term treatment with lithium

Next, we sought to determine presynaptic characteristics and network activity under long-term lithium treatment over a period of 21 days. In order to assure that cell viability was maintained throughout the course of experiments, spectrophotometric analysis of cytosolic lactate dehydrogenase (LDH) in culture supernatants were performed. Control and lithium treatment yielded comparable values (mean LDH activity for lithium 0.090 ± 0.015 [a.u.] versus control 0.094 ± 0.021 [a.u.]) indicating that chronic lithium administration does not impair cell integrity (Figure 3B). In parallel, lithium concentrations were validated before and after the 21 days of incubation and determined to be approximately 1 mM (mean before 0.942 ± 0.025 [mM] versus 0.992 ± 0.035 [mM] after incubation, Figure 3C), thus ruling out the possibility of varying drug concentrations, e.g. elevation due to evaporation of water during the three

weeks of lithium treatment or the antibody incubation.

To analyze effects of long-term lithium administration on synapse formation, sizes and network characteristics, we performed functional antibody labeling experiments on endogenous synaptotagmin1 (Syt1) by using anti-Syt1-CypHer5-antibodies (Adie, Kalinka et al. 2002; Welzel, Henkel et al. 2011). As opposed to pHluorins, the pH-sensitive cyanine dye CypHer5 is non-fluorescent when exposed to neutral pH and fluorescent in acidic environments. Presynaptic labeling was achieved by endocytosis of anti-Syt1-CypHer5 labeled endogenous Syt1 during 1 hour of incubation without any stimulation (Figure 3A). Multipoint acquisition with a pattern of 10x10 images yielded large images of marked active synapse terminals reflecting the neuronal network (Figure 3D). Previously developed MATLAB algorithms (Welzel, Tischbirek et al. 2010) allowed us to detect synapses automatically within this network and to determine their number of neighbors in a defined circular area of 50 μm^2 (Figure 3E). While obtained values for median brightness of neighbors exhibited a trend towards lower brightness values (median 6332 ± 846 [a.u.] under lithium treatment versus 7880 ± 203 [a.u.] under control treatment, students t-test p=0.13, n=6 experiments (or fields of view)?), the number of neighbors was comparable between lithium treated cells (mean 3.14 ± 0.12) and cells treated with control saline (mean 3.29 ± 0.10, Figure 3F). Although the different brightness levels were not significant, it may indicate decreased recycling rates or increased release probability that lead to smaller recycling pool sizes of presynaptic terminals upon chronic lithium administration.

To address the possibility of smaller recycling pool sizes, we performed experiments using the styryl dye FM4-64. We stimulated cells on days 6, 13 and 20 under chronic lithium treatment with a strong stimulus of 1200 AP at 30 Hz that is sufficient to cause depletion of vesicles from the recycling pool (Ryan and Smith 1995). Subsequent compensatory endocytosis traps FM4-64 inside retrieved vesicles and fluorescence intensity after washing as well as mean fluorescence difference after unloading provide a good estimate of the amount of exocytosis (Gaffield and Betz 2006). Analysis of captured images and calculated mean ΔF values for synapses as a measure of the recycling pool size were plotted over time (Figure 3G). Since the values were not significantly increased or decreased for each individual time point, these data indicate that chronic lithium treatment over 21 days with clinically relevant concentrations does not affect the recycling pool size of hippocampal neurons *in vitro* or the size of the presynapse, which correlates with the number of vesicles (Pierce and Mendell 1993).

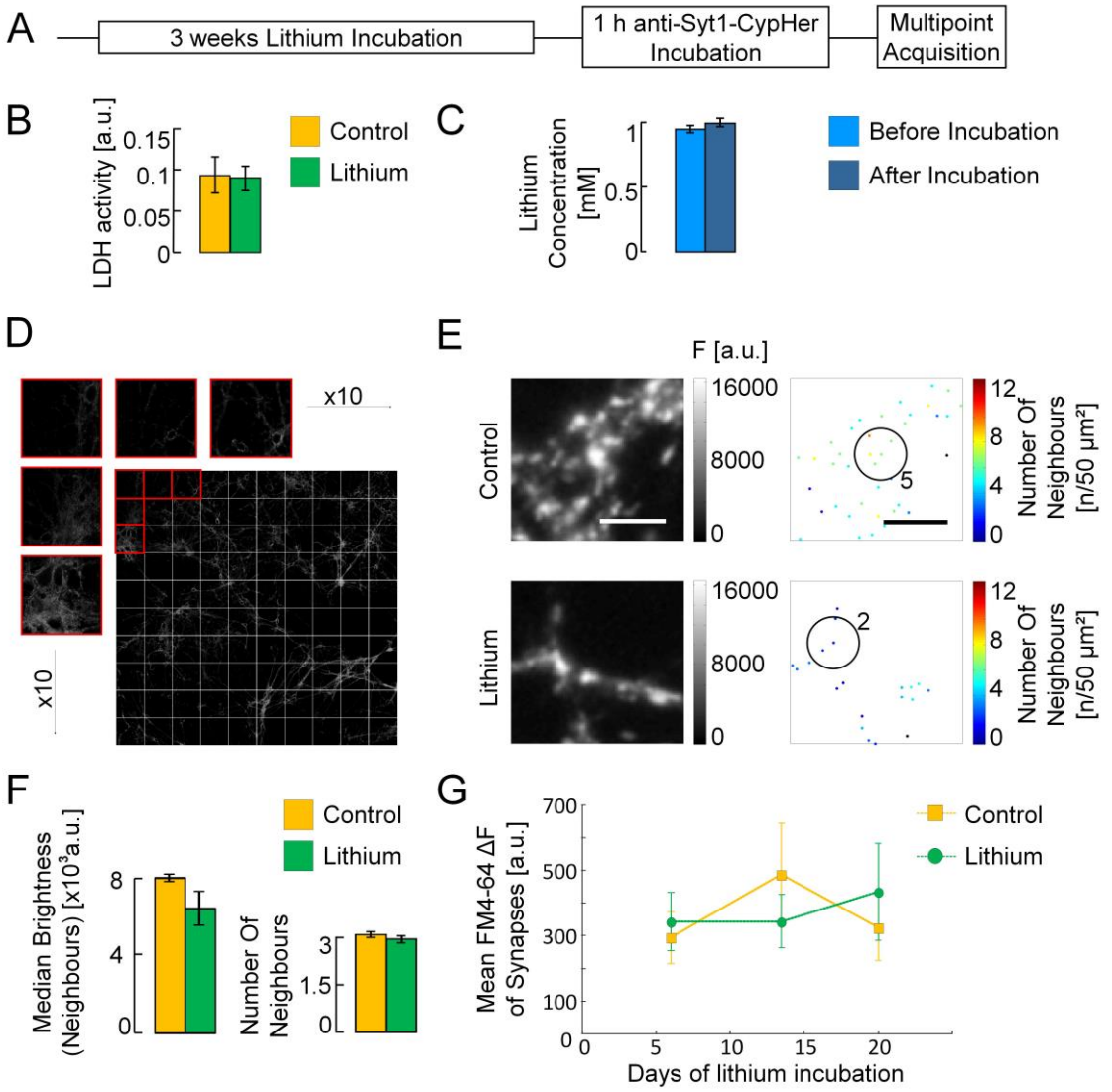

Figure 3 Chronic lithium treatment does not affect synaptic clustering.

A Scheme of the experimental procedure: Rat hippocampal neurons were treated with lithium chloride or sodium chloride for 3 weeks. Presynaptic terminals were labeled with anti-Syt1-CypHer. **B** Spectrophotometric LDH activity analyses were performed after three weeks of lithium incubation. **C** Lithium concentrations as determined before and after anti-Syt1-CypHer incubation. **D** Representative 10x10 large image reflecting the neuronal network of labeled synapses. **E** Exemplary fluorescence images (to illustrate different neighbor number situations (Five and two neighbours) of control and lithium treated hippocampal neurons, respectively) with calculated analytical images: Positions of computationally detected synapses are represented by dots and colour-coded according to their number of neighbouring synapses in a defined circular area of 50 µm^2. Scale bars, 10 µm. **F** Quantitative analysis of gathered data and calculation of mean brightness levels and number of neighbours. (n=6 experiments). **G** Mean ΔF from FM4-64 experiments in hippocampal neuronal culture under lithium treatment for three weeks. The dye was loaded (1200 AP, 30 Hz) and imaged at day 6, 13 and 20. (n=3 experiments, 500-700 boutons were analyzed per treatment). Error bars represent SEM.

Discussion

Among several putative mechanisms of action of therapeutic lithium that have been investigated, the "inositol-depletion hypothesis" (reviewed in (Harwood 2005)) and glycogen synthase kinase 3-mediated effects (Goold, Owen et al. 1999; Hall, Lucas et al. 2000; Tyagarajan, Ghosh et al. 2011) have probably been discussed most intensively. In either case, observations suggest that the effects of lithium occur due to enzyme inhibition with multiple downstream effects. With the notion that lithium effects are not linked to a sole pathway and in light of the growing interest in presynaptic pathology of psychiatric diseases (Wang, Su et al. 2003; Tischbirek, Wenzel et al. 2012) we sought to study functional consequences of acute as well as chronic lithium treatment on presynaptic processes in hippocampal neuronal culture. The choice of the culture was motivated by the fact that postsynaptic effects of lithium have been well demonstrated in this system and by that presynaptic vesicle recycling is commonly measured in hippocampal tissue culture. Also, the hippocampus (Blumberg, HP et al. Arch Gen Psych, 2003) is reduced in size in patients with bipolar disorder. The fact that the reduction of amygdala volumes is however more pronounced in these patients is an important limitation for the interpretation of our study. We were surprised to demonstrate that short-term treatment with both high and clinically relevant concentrations does not change activity dependent or spontaneous vesicle turnover. Consistently, long-term treatment with lithium in a concentration of 1 mM did not affect recycling of vesicles, spontaneous network activity and vesicle pool sizes.

Regarding the inositol depletion hypothesis, it seems remarkable that exo- and endocytosis, which are regulated by phosphoinositides (Milosevic, Sorensen et al. 2005; Antonescu, Aguet et al. 2011; van den Bogaart, Meyenberg et al. 2011; van den Bogaart, Meyenberg et al. 2012), were not altered. This might be explained by other inositol supplies or the observation that inositol is only reduced by 30% (Allison and Stewart 1971). Lower intracellular inositol levels may still give rise to sufficient concentrations of PIP_2, as demonstrated in an inositol transporter-deficiency-induced inositol depletion model (Berry, Buccafusca et al. 2004), and PIP_2 microdomains may still be maintained.

It is also remarkable that acute administration of lithium concentrations of even 40 mM, which are 40-fold higher than therapeutically used concentrations, did not alter the rate of vesicle release upon stimulation, nor the vesicular endocytosis, which appears to be more sensitive to ion fluctuations (Mansvelder and Kits 1998). Using synaptopHluorin both altered exo- and endocytosis would have been readily detectable as to the means of peak detection and determination of the endocytosis constant τ. The former has been shown to yield increased fluorescence values when endocytosis is inhibited (Fernández-Alfonso and Ryan 2004) and the latter provides a good estimate of endocytosis kinetics. A disadvantage of the mere amplitude determination is however, that it could in principle fail in detecting changes compensated by the compound (exo- and endocytic) nature of the signal. On the other hand, it allows to repeatedly measuring the parameter. Together, these data emphasize that lithium does not interfere with these dynamic processes.

Finally, regarding the etiology of bipolar disorder, cell loss and atrophy of both neurons and glia, and abnormalities in cellular plasticity cascades have been suggested to be implicated (Schloesser, Huang et al. 2008). This is in accordance with the 'synaptogenic hypothesis' that was recently suggested as a model for depression (Duman and Aghajanian 2012). The essence of this hypothesis is that

decreased numbers of neuronal synapses in disease-associated areas underlie aberrant information processing and atypical activation of key mood-related circuits. We did not find any difference in spontaneous network activity and the number of active synapses by neighbor analysis of synapses in lithium-treated cells compared to controls. Vesicle pool sizes as determined by total recycling pool labeling were also unaltered, consistently with unaltered neighbor numbers arguing against presynaptic plasticity alterations in terms of increased or decreased synapse numbers or sizes. This is especially interesting as recently reported postsynaptic remodeling (Tyagarajan, Ghosh et al. 2011) appears not to be mirrored by functional presynaptic changes. Functional information on the level of circuits, however, cannot be determined in hippocampal neurons *in vitro*. Considering our data in light of reports on effects of lithium on growth cones (Goold, Owen et al. 1999; Hall, Lucas et al. 2000), which are morphological correlates of synapse development, lithium might not influence the total number of synapses, but rather ameliorate dysregulated circuit connectivity at other functional levels. Elucidating mechanisms of dysregulated transmission in emotion processing that might underlie mood disorders demands for both functional process-related and circuit-based approaches (Price and Drevets 2010) as two complementary parts to investigate a complex pathology. Therefore, our results on synaptic vesicle recycling provide an important basis for the interpretation of other findings.

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